

Overexpression of SIRT1 in urothelial carcinoma of the urinary bladder is associated with local recurrence and poor survival

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ABSTRACT

الأهداف: تقييم العلاقة ما بين اعتصار السيرت ١ (SIRT1) بالصبغات المناعية و العلامات الإكلينيكية في سرطان المثانة البولية.

الطريقة: شملت الدراسة 147 عينة، 122 عينة لسرطان المثانة البولية و25 عينة للأنسجة الطبيعيه المحيطه بالسرطان. العينات جمعت قبل التدخل العلاجي الموضعي والنظامي. مصفوفة الأنسجه صممت وعملت وقطعت لعمل الصبغات المناعية تم عملها باستخدام مضادات السيرت ١.

النتائج: في سرطان المثانة البولية اعتصار السيرت ١ العالي (59.8%) أعلى من اعتصار السيرت ١ المنخفض (40.2%). زيادة اعتصار سيرت ١ في سرطان المثانة البولية مصاحب بعودة حدوث المرض المحلي. ليس هناك ارتباط بالعلامات الإكلينيكية الأخرى في سرطان المثانة البولية. أظهر تحليل الانحدار أن اعتصار السيرت ١ العالي SIRT1 عامل متنبيء مستقل لعودة المرض المحلي. زيادة اعتصار سيرت ١ SIRT1 في سرطان المثانة البولية مصاحب بفترة بقاء قصيرة على قيد الحياة، رتبة السجل (Mantel-Cox) = 6.478 و p=0.011) و بفترة بقاء قصيرة على قيد الحياة خاليه من المرض (log rank [Mantel-Cox]=4.281 و p=0.039).

الخاتمة: نتائج الدراسة أظهرت أن اعتصار السيرت ١ SIRT1 عامل نذير مستقل محتمل لمرضى سرطان المثانة البولية وأنه هدف محتمل للتدخل العلاجي. المطالبه بالمزيد من الأبحاث الجزيئية والسريرية لاستكشاف المصب الجزيئي للسيرت ١ SIRT1 في سرطان المثانة البولية.

Objectives: To investigate the relationship of Silent mating type information regulation 2 homolog-1 (SIRT1) immunostaining to urothelial carcinoma of the urinary bladder (UCB) clinicopathological parameters.

Methods: The study includes a total of 147 specimens composed of 122 urothelial carcinoma and 25 of non-neoplastic normal mucosae. The clinical information and the corresponding paraffin blocks of the cases were collected from the Pathology Department at

King Abdulaziz University, Jeddah, Kingdom of Saudi Arabia. Tissue microarrays were prepared and unstained slides were cut from the recipient blocks. Immunohistochemistry study was performed using anti-human SIRT1 antibody. The study was conducted from July 2016 until May 2018.

Results: In UCB, high SIRT1 immunostaining (59.8%) was greater than low SIRT1 immunostaining (40.2%). High SIRT1 immunostaining was associated with local disease recurrence ($p=0.017$). However, there was no relation with other clinicopathological parameters. Regression analysis demonstrated that SIRT1 overexpression is an independent predictor of local disease recurrence ($p=0.002$). High SIRT1 immunostaining was associated with lower overall survival (log rank [Mantel-Cox]=6.478, and $p=0.011$) and disease-free survival (log rank [Mantel-Cox]=4.281, and $p=0.039$).

Conclusion: The results revealed that SIRT1 is an important prognostic factor for UBC patients and is a potential target for therapeutic intervention. Further immunohistochemical and molecular evaluations are required to explore the mechanism of action of SIRT1 and to investigate molecular downstream of this potential biomarker in UCB.

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Urothelial carcinoma of the urinary bladder (UCB) is a common cancer worldwide.¹ In Kingdom of Saudi Arabia, UCB is listed among the top 10 common cancers in males.² Urothelial carcinoma of the urinary bladder categorized pathologically as low grade and high grade. Usually low-grade neoplasms are associated with good prognosis after transurethral resection while patients with high-grade tumors require more aggressive management. Tumor recurrence is a common problem in UCB patients and occurs in approximately 70% of non-muscle invasive UCB patients following treatment. Non-muscle invasive tumor patients may develop muscle invasion (around 15%).³ Histologic grade is an important prognostic factor for recurrence and progression in non-invasive tumors. The high rate of recurrence in UCB require constant life time follow-up which results in a high cost of management.⁴ Silent mating type information regulation 2 homolog-1 (SIRT1) is a key member of the sirtuin family.⁵ Silent mating type information regulation 2 homolog-1 plays an important role in many physiological processes such as cell metabolism, aging, apoptosis, proliferation and tumorigenesis.^{5,6} Silent mating type information regulation 2 homolog-1 can serve as either a tumor promotor or tumor suppressor depending on the oncogenic pathway for different types of tumors.⁷ Overexpression of SIRT1 was detected in many human cancers such as non-small cell lung cancer, oesophageal squamous cell carcinoma, prostate, hepatocellular, colon, gastric, ovarian, breast, pancreatic, and endometrial cancers.⁸⁻¹⁷ However, the SIRT1 expression pattern in UCB is unclear, and the role of SIRT1 in UCB remains unknown. The objective of this study was to evaluate the relationship of SIRT1 immunopexpression to various clinicopathological parameters and the value SIRT1 as a predictor of the disease outcome in UCB Saudi patients.

Methods. The study includes a total of 147 specimens composed of 122 urothelial carcinoma and 25 of non-neoplastic normal mucosae. The clinical information and the corresponding paraffin blocks of the cases were collected from the Pathology Department at King Abdulaziz University, Jeddah, Kingdom of Saudi Arabia. All pathological specimens that were used in the study were collected before initiation of local and systemic therapy. Tumor stages were reviewed according to cancer staging atlas of the

American Joint Committee On Cancer.¹⁸ Tumor grades were revised according the World Health Organization's (WHO) classification.¹⁹ Clinicopathological data are summarised in **Table 1**. The study was approved by the Biomedical Research Committee, Faculty of Medicine, King Abdulaziz University in Jeddah, Kingdom of Saudi Arabia. All patients were consented to use their pathological specimens for laboratory investigations which is a routine practise at King Abdulaziz University Hospital in Jeddah, Kingdom of Saudi Arabia.

Table 1 - Clinicopathological parameters of tumors (n=122).

Parameters	n (%)
<i>Gender</i>	
Male	101 (82.8)
Female	21 (17.2)
<i>Age</i>	
<60 years	46 (37.3)
≥60 years	76 (62.3)
<i>Grade</i>	
Low grade	32 (26.2)
High grade	90 (73.8)
<i>Muscle invasion</i>	
Negative	50 (41)
Positive	72 (59)
<i>Pathological stage (pT)</i>	
T1	59 (48.4)
T2	44 (36.1)
T3	8 (6.6)
T4	11 (9)
<i>Nodal metastasis</i>	
Negative	98 (80.3)
Positive	98 (80.3)
<i>Distant metastasis</i>	
Negative	109 (89.3)
Positive	13 (10.7)
<i>Lymphovascular invasion</i>	
Negative	101 (82.8)
Positive	21 (17.2)
<i>Anatomical stage</i>	
I	56 (45.9)
II	32 (26.2)
III	4 (3.3)
IV	30 (24.6)
<i>Local disease recurrence</i>	
Negative	82 (67.2)
Positive	40 (32.8)
<i>Survival</i>	
Alive	86 (70.5)
Dead	36 (29.5)

T1 - tumor invades subepithelial connective tissue, T2 - tumor invades muscularis propria, T3 - tumor invades perivesical tissue, T4 - tumor invades any of the following: prostatic stroma, seminal vesicles, uterus, vagina, pelvic wall, or abdominal wall, stage I - (T1, N0, M0), stage II - (T2, N0, M0), stage III - (T3 or T4a, N0, M0), stage IV - (any T, N1-3 or M1).

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Tissue microarray. The technical preparation of the tissue microarrays (TMA) was carried out as previously described.²⁰ The haematoxylin and eosin slides were reviewed and the selected areas of the tumor or the normal mucosa were marked. The corresponding tissue in the donor blocks was utilized to get 2 tissue cores and transferred to the recipient blocks by using the automated tissue arrayer (Master 3D Histech, Budapest, Hungary). Unstained slides were prepared from TMA recipient blocks and used for immunohistochemistry study.

Immunohistochemistry. Immunostaining was carried out anti-SIRT rabbit polyclonal antibody (H-300: sc-15404 from Santa Cruz Biotechnology Inc.). Immunohistochemistry study was performed using an automated system (Ventana Bench Mark XT, Ventana Inc., Tucson, AZ). Colorectal carcinomas known to be positive for anti-SIRT1 antibody were used as positive controls. Negative controls were processed without primary antibody.

Evaluation of SIRT1 immunostaining. A semiquantitative SIRT1 immunostaining scoring was performed depending on percentage and intensity of nuclear staining. Percentage was expressed as 5 categories (0=0%, 1=1-10%, 2=11-30%, 3=31-50%, and 4=51-100%) while Staining intensity was divided into 4 categories (0=negative; 1=weak 2=moderate, and 3=strong). For each the sum of intensity and percentage

were calculated and a final score from 0-7 was obtained. The final score was dichotomized in which a sum of 0-3 was considered low SIRT1 immunostaining while 4-7 was considered high SIRT1 immunostaining.

Statistical analysis. The chi-square test was used for testing the differences between 2 groups of variables. The overall and disease-free survivals were measured by the Kaplan-Meier method with the log-rank (Mantel-Cox) comparison test. Disease-free survival was calculated as the time from diagnosis to the appearance of recurrent disease (or date of the last seen disease-free appearance). In order to test the prognostic significance of SIRT1 immunostaining as a predictor factor, a binary logistic regression analysis was used. The estimated odds ratio (exponential B [exp B]), 95% confidence interval (CI) for exp B were expressed for each regression analysis. Statistical analyses were performed using the Statistical Package for the Social Science (SPSS®) software packages version 16 (SPSS Inc., Chicago, IL, USA). A *p*-value<0.05 was considered significant.

Results. Pattern of SIRT1 immunostaining. Silent mating type information regulation 2 homolog-1 nuclear immunostaining was detected in apparently normal urothelium adjacent to UCB (Figure 1A). Low SIRT1 immunostaining was observed in 80% of the normal epithelium, while high immunostaining was found in 20%. In UCB, high SIRT1 immunostaining (59.8%)

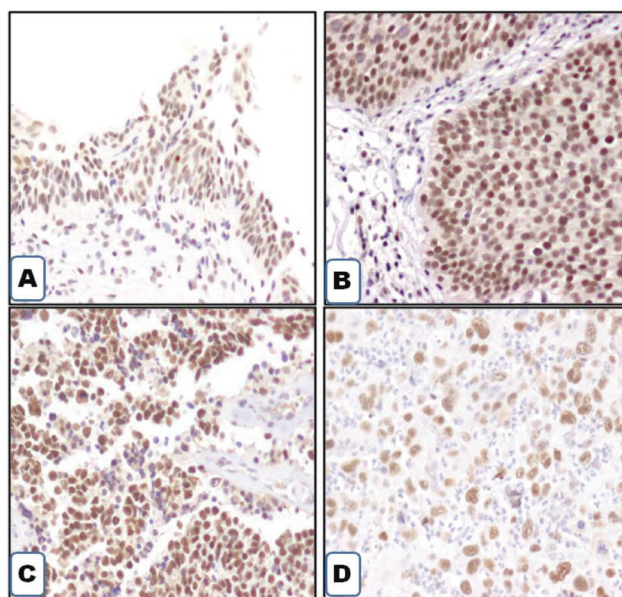


Figure 1 - SIRT1 immunostaining. A) A section from a normal urinary bladder mucosa shows low nuclear silent information regulator (SIRT1) immunostaining (200x). B-D) Sections from urothelial carcinomas show high nuclear SIRT1 immunostaining in malignant urothelial cells (100x). Immunohistochemistry was carried out using an anti-SIRT1 antibody and diaminobenzidine as the chromogen and haematoxylin as a counterstain.

was more prevalent than low SIRT1 immunostaining (40.2%). Silent mating type information regulation 2 homolog-1 immunostaining in malignant urothelial cells is shown in **Figure 1B, 1C, and 1D**. The level of SIRT1 immunostaining in UCB and in apparently normal urothelium is shown in **Table 2**.

Distribution of SIRT1 immunostaining in relation to prognostic factors of UCB. The distribution of SIRT1 immunostaining among clinicopathological parameters is shown in **Table 3**. High SIRT1 immunostaining was associated with local disease recurrence ($p=0.002$). However, there was no association with the other clinicopathological parameters. Regression analysis revealed that high SIRT1 immunostaining is an independent predictor of local disease ($p=0.002$, Exp B=0.250, 95% CI: [0.103-0607]).

Survival outcome in relation to SIRT1 immunostaining. In UCB, high SIRT1 immunostaining was associated with lower overall survival (log rank [Mantel-Cox]=6.478 and $p=0.011$) and disease-free survival (log rank [Mantel-Cox]=4.281 and $p=0.039$) **Figures 2&3**.

Discussion. The human genome encodes 18 histone deacetylase (HDAC) genes. Histone deacetylases are classified into 4 families (classes I-IV). Classes I, II, and IV are zinc-dependent enzymes and labelled as HDACs, while class III, HDACs need nicotinamide adenine dinucleotide (NAD⁺) for enzymatic activity and is labelled as a sirtuin.²¹ Silent mating type information regulation 2 homolog-1 is a key member of the sirtuin family.⁵ The acetylation/deacetylation cycle of proteins is important in the process of gene expression and regulation.²² Silent mating type information regulation 2 homolog-1 was found to be associated with poor prognosis, advanced stages, and shorter patient survival in many human cancers. Overexpression of SIRT1 was also found to be associated with advanced pathological tumor stage, poor differentiation, shorter recurrence-free survival in non-small cell lung cancer, higher tumor stage, poor outcome in esophageal squamous cell carcinoma, tumor stage, tumor differentiation, shorter overall and relapse free survivals in gastric cancer, shorter survival in ovarian cancer, higher tumor stage

in hepatocellular carcinoma, lymphovascular space invasion worse prognosis in colorectal cancer patients, shorter survival and aggressive behaviour in endometrial carcinoma, high tumor stag, lymph node metastasis, and worse clinical outcomes in breast cancer.^{8,9,11-14,17,23-25}

On the other hand, some studies revealed that SIRT1 levels were significantly lower in patients with cancer than normal tissue such as oral squamous cell carcinoma.²⁶ Kang et al.²⁶ demonstrated that SIRT1 overexpression inhibited cancer cell invasion and proliferation and suggested that SIRT1 is a tumor suppressor gene in oral squamous cell carcinoma. Similar results were also demonstrated in renal cell carcinoma.²⁷

There are limited studies that evaluated the status of SIRT1 in UCB. These data are predominantly limited to the evaluation of SIRT1 expression in UCB cell lines.²⁸⁻³¹ Previous studies showed that the level of SIRT1 immunostaining is statistically higher than that of apparently normal urothelium.^{30,32,33} In this study, it was demonstrated that high SIRT1 immunostaining is associated with local disease recurrence, and regression analysis showed that high SIRT1 immunostaining is an independent predictor of local disease recurrence. Local disease recurrence is a common problem in UCB. Despite the radical surgical approach for UCB, the recurrence rate varies from 22-70%.^{3,34,35} A recent study revealed that 70% of these recurrences occurred in the first year following surgery.³⁴ There are known risk factors for UCB recurrence including tumor multiplicity, tumor stage and operative procedure; however, recognition of further independent predictor of tumor recurrence may be helpful in management plan.

It was also demonstrated in this study that high SIRT1 immunostaining was associated with lower overall and disease-free survivals in UCB patients. However, in this study there was no association with other clinicopathological parameters including gender, age, tumor grade, muscle invasion, pathological stage, nodal and distant metastases, and lymphovascular invasion. Hu et al.³⁰ found that there is upregulation of SIRT1 in cancer tissues when compared with

Table 2 - Categories of SIRT1 immunostaining in urothelial carcinoma of the urinary bladder (UCB) and normal urothelium.

Histopathological category	Low immunostaining	High immunostaining	P-value
Primary tumor (n=122)	49 (40.2)	73 (59.8)	0.882
Normal urothelium (n=25)	20 (80)	5 (20)	

SIRT1 - Silent mating type information regulation 2 homolog-1

Table 3 - The distribution of SIRT1 immunostaining and clinicopathological features of UCB.

Parameters	SIRT1 Immunostaining		P-value
	Low	High	
Gender			
Male	42	59	0.483
Female	7	14	
Age			
<60 years	19	27	0.842
≥60 years	30	46	
Grade			
Low grade	11	21	0.437
High grade	38	52	
Muscle invasion			
Negative	19	30	0.965
Positive	26	37	
Pathological stage (pT)			
T1	22	34	0.940
T2	12	20	
T3	2	2	
T4	13	17	
Nodal metastasis			
Negative	40	58	0.766
Positive	9	15	
Distant metastasis			
Negative	43	66	0.641
Positive	6	7	
Lymphovascular invasion			
Negative	40	61	0.782
Positive	9	12	
Anatomical stage			
I	23	36	0.913
II	17	27	
III	4	4	
IV	5	6	
Local disease recurrence			
Negative	41	41	0.002
Positive	8	32	

T1 - tumor invades subepithelial connective tissue, T2 - tumor invades muscularis propria, T3 - tumor invades perivesical tissue, T4 - tumor invades any of the following: prostatic stroma, seminal vesicles, uterus, vagina, pelvic wall, or abdominal wall, stage I - (T1, N0, M0), stage II - (T2, N0, M0), stage III - (T3 or T4a, N0, M0), stage IV - (any T, N1-3 or M1).

paracancerous and normal bladder tissues. By establishing a SIRT-1 knockdown UBC model, Hu et al,³⁰ concluded that proliferation and viability were suppressed with SIRT1 deficiency in UCB cells. However, no evaluation of the relation between SIRT1 expression and clinicopathological parameters was carried out in that study.

The dual nature of SIRT1 in human cancer remains variable, and these variable results could reflect different roles of SIRT1 in different organs. However, the different expression patterns could also be due to several

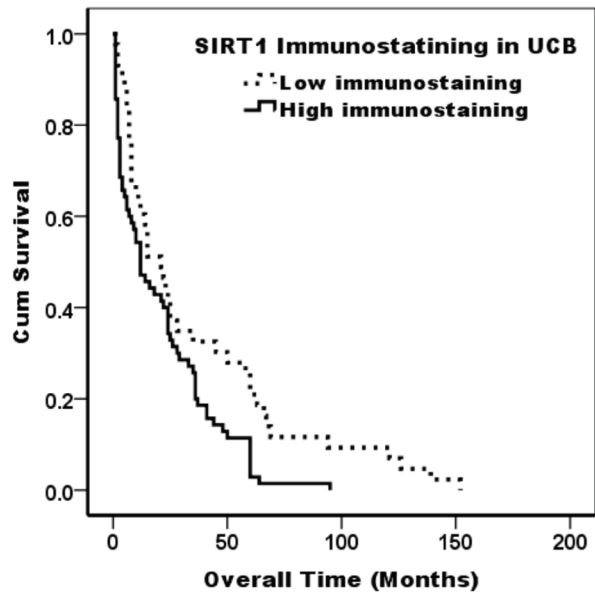


Figure 2 - Overall survival curve (Kaplan Meier) according to SIRT1 immunostaining. There is a difference in survival probability between low and high SIRT1 immunoexpression (log-rank=6.478, $p=0.011$).

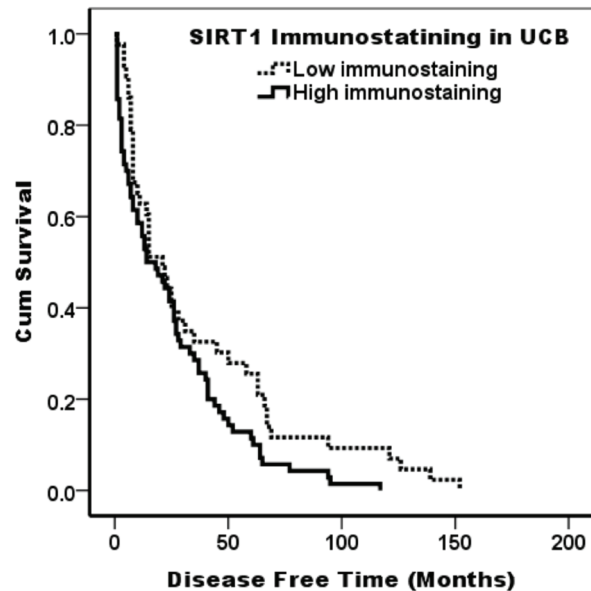


Figure 3 - Disease-free survival curve (Kaplan Meier) according to SIRT1 immunostaining. There is a difference in survival probability between low and high SIRT1 immunoexpression (log-rank = 4.281, $p=0.039$).

factors, including its subcellular location and diverse downstream substrates.^{36,37} In a meta-analytical study, Wang et al,³⁶ reviewed 37 studies including a total of

7,369 cases of solid tumors and concluded that SIRT1 was expressed in 48.6% of the patients and SIRT1 overexpression was significantly associated with overall survival and poor prognosis. The current study supports that SIRT1 serve as tumor promotor in UCB.

The mechanism of SIRT1 function is complex and conducted through p53, forkhead box protein (Fox) O1, nuclear factor (NF)- κ B, and other signalling pathways.³⁶ Many studies have shown that the possible regulatory mechanism of SIRT1 as a cancer gene is associated with tumor protein p53.³⁸ Silent mating type information regulation 2 homolog-1 plays a significant role in tumorigenesis.³⁶ Few studies have evaluated the role of other histone deacetylases in UCB. It has been demonstrated that high HDACs expression is present in UCBs and it has been found that HDACs were significantly associated with higher tumor grade and patient survival.^{39,40}

In conclusion, the current study demonstrated that high SIRT1 immunoexpression is an independent predictor of local disease recurrence. High SIRT1 immunostaining is associated with lower overall survival and disease-free survival. The current results showed that SIRT1 is a potential independent prognostic factor for UCB patients and could contribute to UCB progression. The SIRT1 is a potentially promising target for therapeutic intervention as a post-operative treatment for UCB. The results warrant further investigations into the specific functions of SIRT1 in UCB and exploration of the molecular downstream events associated with SIRT1.

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